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|--|----------------------|-------------------|
| DECLARATION OF JOSEPH FISHER UNDER 37 C.F.R. §1.131 | Application Number | 09/293,670 |
| | Confirmation Number | 5176 |
| | Filing Date | April 16, 1999 |
| | First Named Inventor | Joseph Fisher |
| | Examiner | Teresa Wessendorf |
| | Group Art | 1639 |
| | Attorney Docket No. | RIGL-036CIP |

This Declaration with the attached Exhibits are being submitted in conjunction with the Applicants' Response to the Office Action dated February 24, 2006.

I, Joseph Fisher, M.D. Ph.D. do hereby declare as follows.

1. I am listed as an inventor of the above-referenced patent application.
2. Between June and September, 1997, I was a Scientist at Rigel Pharmaceuticals, Inc. (hereinafter "Rigel"). During this time, I was part of a program focused on the discovery of intracellularly-active peptides. The strategy employed by this program involved infecting cells with a library of retroviral vectors encoding candidate peptides, and selecting cells with an altered phenotype using fluorescence activated cell sorting (FACS)-based methods. The idea of using more than five FACS parameters to identify retrovirally-delivered, intracellularly-active peptides was developed before July 31, 1997.
3. I understand that the claimed subject matter of the above-referenced patent application relates to screening methods that include sorting a population of retrovirally infected cells using at least five fluorescence activated cell sorting (FACS) parameters. I


have been asked to provide factual evidence relating to my activities at Rigel with respect to the claimed subject matter before and after July 31, 1997.

4. Experiments confirming the applicability of FACS-based screening methods that employ at least five FACS parameters to the discovery of retrovirally-delivered intracellularly-active bioactive peptides were performed prior to July 31, 1997.
5. Exhibit A, which is a copy of pages 24 and 25 of my laboratory notebook, describes the results of an experiment in which cells were treated to induce exocytosis, and sorted using five FACS parameters. Exhibit A is dated prior to July 31, 1997. The top four graphs of page 25 show FACS results obtained from DMSO-treated cells (control), and the bottom four graphs of page 25 show FACS results obtained from A23187-treated cells (experimental). The top left graph of each group of four graphs shows results obtained from the parameter used to detect FM143, a fluorescent dye. The top right graph of each group of four graphs shows results obtained from the parameter used to detect FITC, another fluorescent dye. The bottom left graph shows results obtained from the parameter used to detect propidium iodide. The bottom right graph shows results obtained from parameter used to detect front light scatter as well as, independently, the parameter used to detect side light scatter. Thus, Exhibit A demonstrates the applicability of FACS methods that employ at least five FACS parameters to the discovery of retrovirally-delivered intracellularly-active bioactive peptides, before July 31, 1997.
6. Exhibit B, which is a copy of pages 112 to 120 of my laboratory notebook, describes an experiment in which MC9 and CEM cells are transfected with a library of retroviral vectors that encode peptides. Exhibit B demonstrates that CEM and MC9 cells were transfected with a library of retroviral vectors between August 22 and August 27, 1997.

7. In September 1997, a method that included infecting cells with a library of retroviral vectors encoding candidate bioactive peptides, and selecting cells with an altered phenotype using five fluorescence FACS parameters was reduced to practice.
8. Exhibit C, which is a copy of pages 138 and 139 my laboratory notebook, describes an experiment in which retroviral vector library-infected cells are stimulated staurosporine to induce apoptosis, and sorted using five FACS parameters: side scatter ("ssc"), front light scatter ("fsc"), and three separate fluorescence parameters: ("fl1", "fl2" and "fl3"). Results for control cells not contacted with staurosporine are shown in the graphs on the left hand side of page 139, and results for experimental staurosporine-treated cells are shown in the graphs of the right hand side of page 139. Thus, Exhibit C demonstrates reduction to practice of a method that includes infecting cells with a library of retroviral vectors encoding candidate bioactive peptides, and selecting cells with an altered phenotype using five FACS parameters, on September 8, 1997.
9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: June 25, 2006



Joseph Fisher, M.D. Ph.D.,

Attachments: Exhibits A - C

EXHIBIT A

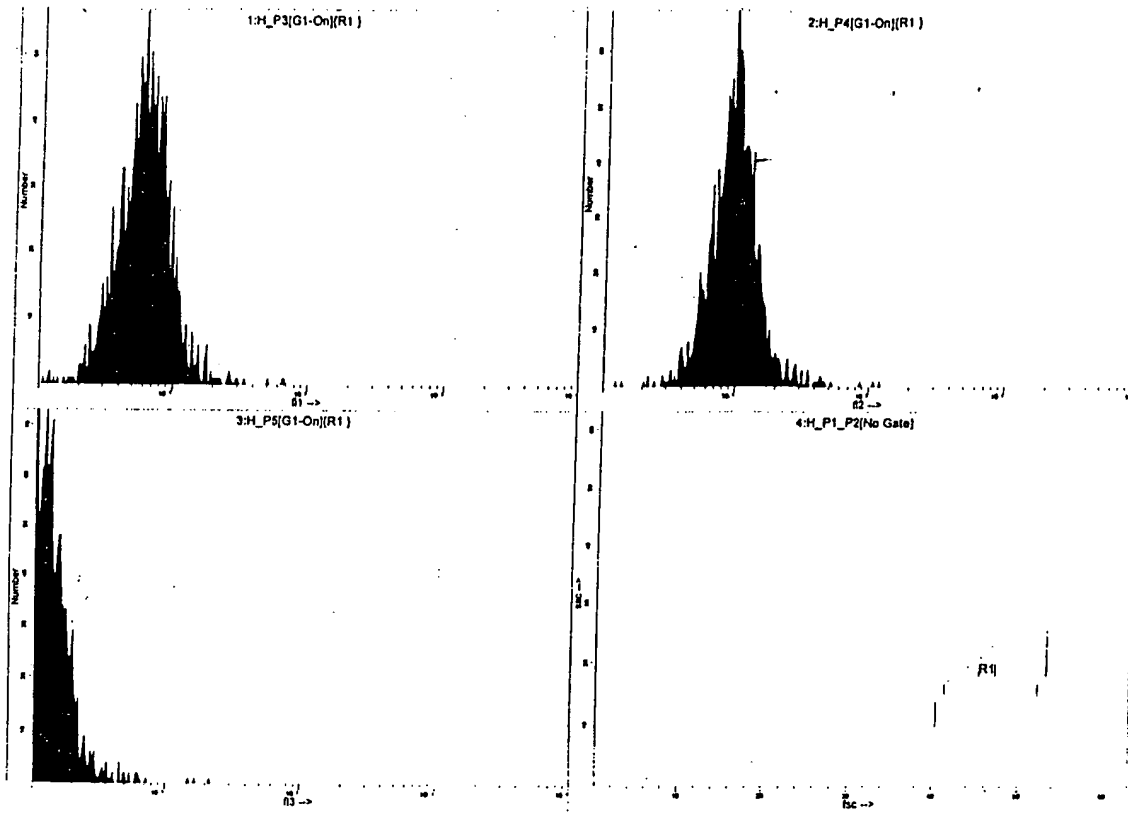
HMC-1 - Exocytosis Trace Dyes

- Try FM-143 and ConA FITC as exocytosis Tracers on HMC-1 cells.
- HMC-1 Cells - From Alexis Spinra ~ 10^6 cells/ml, Highly Viable
- Spin/Wash 5×10^6 cells in MT
- Divide into 2 - $\frac{1}{2}$ Incubate in MT $10'$ 37°C
 - " " " + Succinyl ConA $100 \mu\text{g/ml}$ } $37^\circ\text{C } 10'$
- Wash SCA cells 2x MT
- Take up cells in 1ml MT (no BSA) in 4 tubes
 - A) DMSO } + FM-143 - $2.5 \mu\text{g/ml}$ $\Rightarrow 37^\circ\text{C } 10'$
 - B) + AZ3187 $1 \mu\text{g/ml}$ }
 - C) DMSO } + SCon-A-FITC $25 \mu\text{g/ml}$ \Rightarrow "
 - D) AZ3187 " }

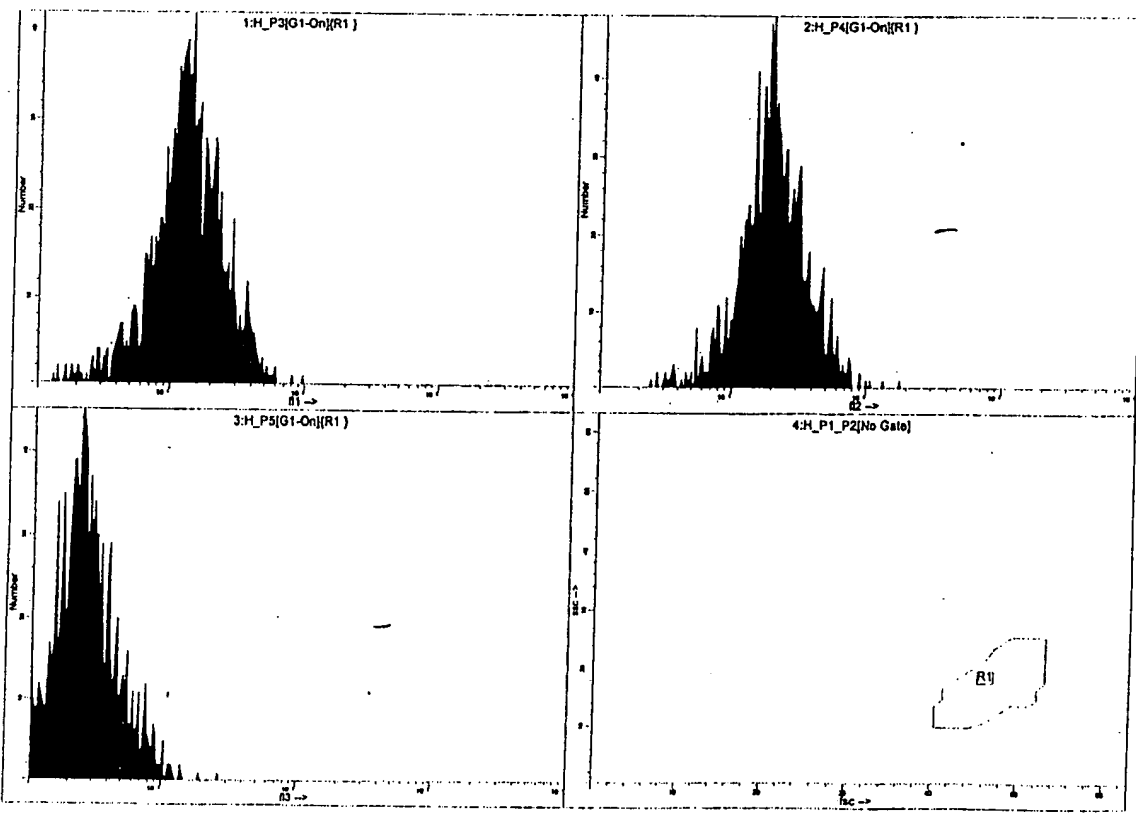
Wash Cells 2x in MT - Take up in 1MT for FACS

Save Files as JMP.DIR.001 } C
 2 } D
 3 } A
 4 } B

n. Page No



0MSP 10'
37°C



1ug/ml
A23187 10'
37°C

To Page No. _____

Assessed & Understood by me,
Ames Loren

Date

Invented by *Josh Ford*
Recorded by

Date

8/22/97

EXHIBIT B

Phoenix E Cell Transfections

⇒ For MC9 Cell Infections

- Use Susans Protocol (x2) So 2 wells of 6 well Plate / Transfection
- DNA - From Jenny Wang

| | | |
|---|-----------------------|-------------------------|
| 1 | (10μg) = 6.6λ | Rab3a and Synaptotagmin |
| 2 | 6.3λ | Constructs |
| 3 | 8.9λ | |
| 4 | 9.1λ | |
| 5 | - New IRES Hook 43-13 | 129.13 10μg = 11.6λ |
| 6 | - " " 6FP 610.25 | 010-25 10μg = 11.1λ |

From Jim L

Randy's Nomenclature

Jim's Nomenclature

- Follow Susans Protocol - Add Precipitate / Chloroquine on cells at 11AM
- Mci Peppy + Precipitate Seen on all Transfectants

Protocol on next page.

7PM

⇒ Aspirate DNA

- Wash 1x in Phoenix Media
- Add 2ml/well Fresh Media

Page No. _____

Protocol for transfection of Phoenix cells and infection of nonadherent target cells**Day 1:**seed Phoenix cells (Es or As) in 6 well plates at 8×10^5 cells in 1.5 ml (DMEM + 10% FBS + P/S) per well**Day 2: CaPO_4 Transfection**

per well: 5 μ g DNA
 30.5 μ l 2M CaCl_2
 219 μ l H_2O
 250 μ l 2X HBS

2 wells

10 μ g DNA
 61 μ l 2M CaCl_2
 438 μ l H_2O
 500 μ l 2X HBS

allow all reagents to come to room temperature 30mins. before starting (do not warm up in H_2O bath)add 50mM chloroquine at 2 μ l/well (50 μ M final)**mix CaPO_4 reagents in 15ml polypropylene tube:**pipet 5 μ g DNA to side of tubepipet 30.5 μ l of 2M CaCl_2 away from the DNAmix the two together with the addition of 219 μ l of miliQ H_2O then using a 1ml pipet, add 250 μ l of 2X HBS and quickly bubble air through the pipet for 2 to 10 secs. (the time is 2 HBS batch dependent)

immediately add mixture dropwise to well

microscopically visible precipitate should appear within a few minutes

incubate 8hrs

remove medium, wash once, and replace with 1.5ml medium

Day3:

move transfected plates to 32°C

Day 4: Infection of target cellscollect virus supernatant from transfected wells (1.5 ml) into 15 ml tubes and add either 1.5 μ l of 5mg/ml polybrene or 1.5 μ l 5mg/ml protamine sulfatecfg out cells and debris at 2500 RPM for 5 mins. or alternatively, filter through .45 μ m acrodisc syringe filtercount target cells and distribute 5×10^5 cells per virus supe to 15ml tubes and pellet 5 mins. 2500 RPM

resuspend each pellet of target cells with virus supe and transfer to one well of a 24 well plate

seal plate with parafilm and cfg at RT for 90 mins. at 2500 RPM

Remove parafilm and incubate plate over night at 32°C

Day 5:

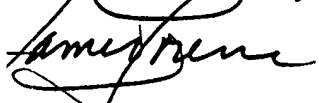
collect and pellet each well of target cells and resuspend in 4ml and transfer each to a 6cm plate

Day 7 or Day 8:

at 48 to 72 hrs. post infection target cells are ready to analyze for expression

To Page No. _____

Assessed & Understood by me,



Date



Invented by



Recorded by

Date

8/22/97

8/23/97

- Transfections of ϕ E Cells - (Cont.)
- This morning. 24 hrs post Transfection Start
Look at Cells by Fluorescence.
GFP \oplus Cells seen in # 3, 4, and 6
3 and 4 must be CT16 Vector (inducible with Ires GFP)
1 and 2 " be resp Hook vector.
- Remove old Media
- Add 2ml/well of Warmed MC9 Media - 12PM

MC9 Positive Control Peptides

MC9 Cells - WT

| | |
|---------------|------------------------|
| Sectam Hook | } ~75% Hook+ From Amy. |
| Synaptotagmin | |
| RAB | |

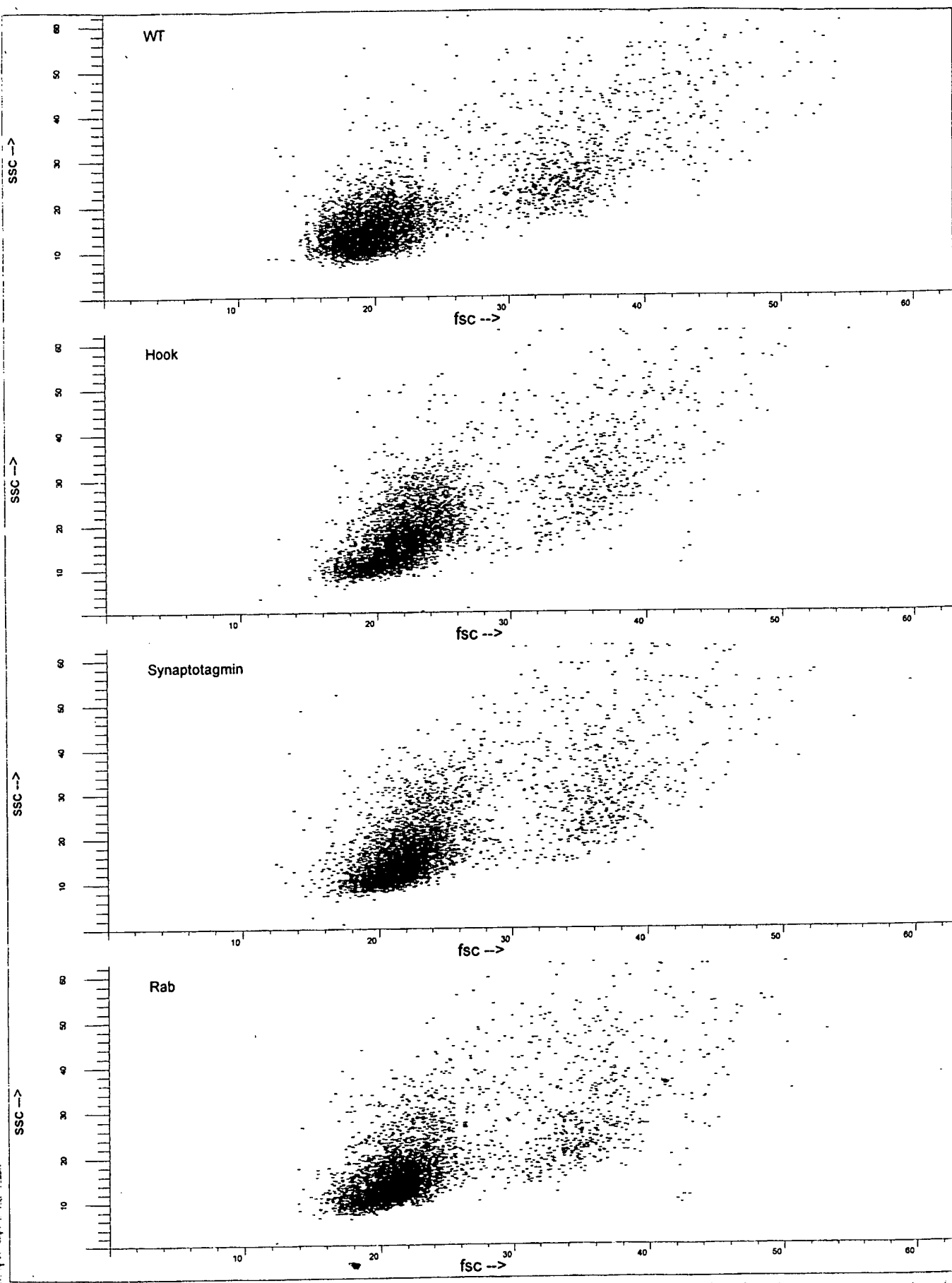
- Aspirate 2ml Cells, Take up in .3ml MT
100x / Tube

\Rightarrow one gets FM143 1mM
 " " " " + 2mM Ionomycin } $37^{\circ}\text{C} \Rightarrow 30'$
 " " PI

View in FACSCAN

- 001 WT
 - 2 Hook
 - 3 Synaptotagmin
 - 4 RAB

5 - WT
 6 + WT
 7 - Hook
 8 + "
 9 - Synaptotagmin
 10 + "
 11 - RAB
 12 + "



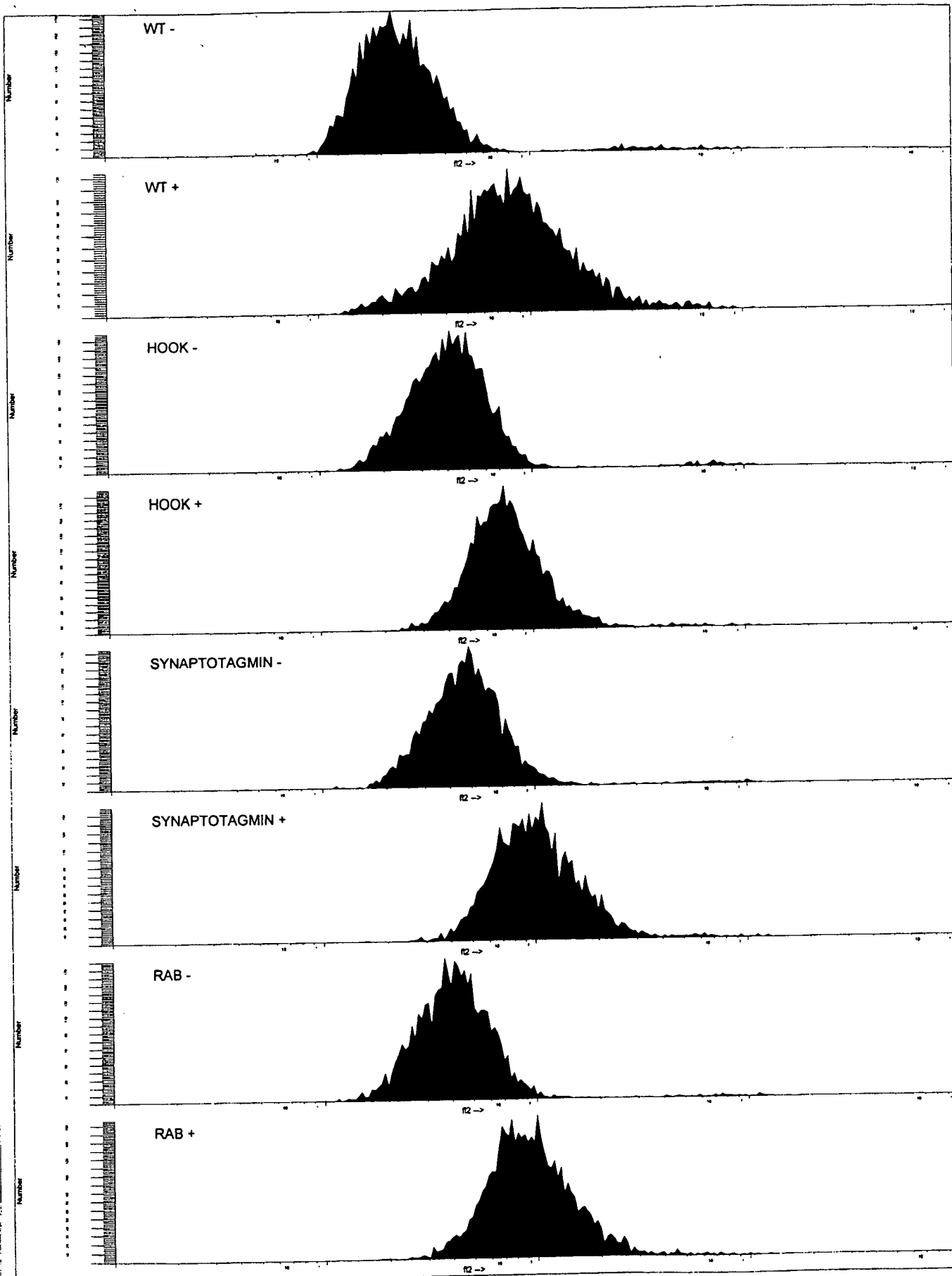
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Invented by
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Recorded by

Date
8/28



Page No. _____

(8/25)

= MC9 Cell Infection (Cont)

- Wells 3/4 + 6 of Transfections Look Significantly Brighter for GFP than they did on 8/23
- ~1 PM - Remove Ural Super - Spin at 2500 RPM x 15' RT
- MC9 Cells, $\sim 2.5 \times 10^6$ /ml
- Spin down 2ml x 6 MC9 cells ($\sim 5 \times 10^6$ /tube)
- Add Ural Super
- Divide Each into 2 wells of a 6 well plate (~ 2 ml / 2.5×10^6 cells/well)
Add 4 μ l of 5mg/ml Polyamine Sulfate / well so FC = 10 μ g/ml
- Seal Plates and Spin for 90' at 2500 RPM
- Culture ON at 37°C ($\sim 3:30$ PM \rightarrow)

- MC9 Cell Harvest - For future cDNA Library ConstructionCells $\sim 2 \times 10^6$ /ml

- Spin down 200ml cells
- Wash 2x in Cold PBS / Asplate
- Freeze on Dry Ice - 2 tubes x 2×10^8 cells/tube
- Store at -80°C

To Page No. _____

Inspected & Understood by me,

Date

8/25/97

Invented by

J. F. L.

Recorded by

Date

8/25/97

8/24/97

MC9 Infection (Cont.)

- 2x 6 well plates infected yesterday
- ~ 11AM, Take cells out of wells/Pool, Wash cells with 2ml MC9 Media, Spin, Decant
- Take up Pellets 1-6 with 12ml MC9 Media and Plate in T-75s
- Quick Look at #6 Shared some GFP⁺ cells.

iresGFP Library Inf. Transfection

- Susan Plated 20 60 mm plates of ϕ E Cells yesterday, today ~ 40% confluent
- Randy Supplied DNA 10-62 Library - 14mer, iresGFP 850 μ g/ml
- For Each 60mm Plate add (Plates have 6ml of media)

82 Chloroquine (50mm)
 10 μ g DNA (11.8 λ)
 122 λ CaCl₂
 876 λ H₂O
 1ml 2x HBS

Transfected 17 Plates From
 11:30AM \rightarrow ~12:30AM

Follow Standard Procedure

~ 6:30PM

- Aspirate Media
- Wash cells 1x in PBS + Ca⁺⁺
- Add Warm MC9 Media - 8 ml / Plate
- 37°C ~ 7PM

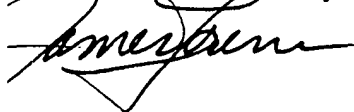
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CEM - Library Infection

Library in resCFP, ~10⁶ Complexity of
 random 14-mer Peptides - Part of 2nd
 ↑ IgE Screen Library.

- Yesterday Susan S. Put CEM Media on Library Infected CPA cells (After She Harvested her virus ~3PM) - Today Renae Supers (~4PM) Spin at 2500 RPM x 10', Add PS to 10µg/ml
- CEM cells, ~1.1 x 10⁶/ml
 Spin Down 60ml (~6.6 x 10⁷ Cells total)
- Divide Pellets into 8 x 12ml Supers → / 8 x T-75s → 8.25 x 10⁶ cells/Flask
 Spin T-75s at 2500 RPM
 4:45 → 6:15
 Take out and Put at 37°C ON

Witnessed & Understood by me,



Date



Invented by



Recorded by

Date

8/26/97

To Page No. _____

Page No. _____

8/27/96

< ϕ E Library Transfection >

A few GFP⁺ cells seen today but a minority
 ~4PM Transfer cells to 32°C

- Split MC9 cells for tomorrow's Infection

[CEM Cells - Library Infection]

~1PM (22 hrs Post Infection Spin) Spin All infected CEM cells - Decant Super
 Take up in 90ml Fresh Media
 Plate in 3x T-150's

- Take out 1ml of Library Infection, 1ml of LT Cells
 Annexin - PE / PI Stain as done on 8/13 (use those controls as well)
 View in FACSCAN

Files 001 LT
 002 Library Infected

→ See next page
 Some GFP⁺ cells showing up in Library Infected after 22 hrs

[Mod MC9 Media]

DMEM (has Pyruvate and Glutamine)

18mg/500ml Asparagine

1x Non-essential AA

0.05mM 2-ME

Pen/Strep 1x

10% HI FBS

10% T-Stim Conditional Media

.2um Sterile Filter

To Page No. _____

Reviewed and Understood by me,



Date

8/26/96

Invented by

Joseph F. M.

Date

8/27/97

9/8/97

CEM-Library Infected - Apoptosis Induction

EXHIBIT C

XX gave me Library Infected Cells to test DNA Rescue Methods - CEM $\sim 2.4 \times 10^6$ /ml
 Take 8ml (2×10^7 cells) + 4ml Fresh Media, Bring to 1ml Stavosporine

→ 37°C 10AM → 4PM (6 Hrs)

→ Annexin PE Stain as usual Procedure = files

.015 GFP ONLY

16 Annexin PE ONLY

17 PI ONLY

18 GFP Library - ϕ Stavo

19 " " + Stavo 7 hrs

new
Settings

CEM-Library - Stavosporine treatment 2x (9/3) - Now 5 days post treatment

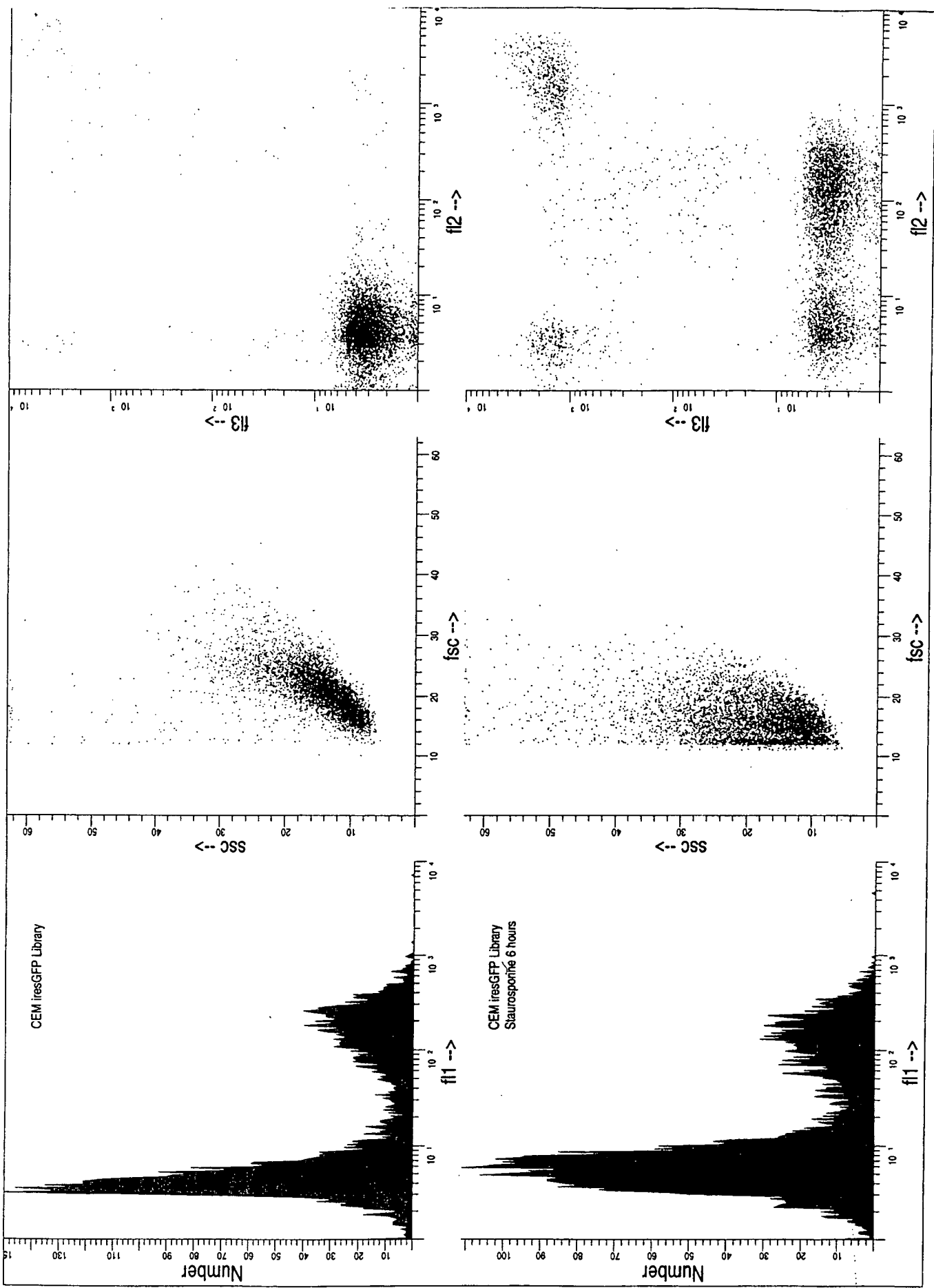
Take .5ml of Culture - Add PI

- FACSCAN - .001 - Library untreated

.002 Treated Stavo 2x

MCF Library - GFP Enriched

- GFP Enriched Cells from last week - now $\sim 2.8 \times 10^6$ /ml $\times 100$ ml
- Split Back to $\sim 10^6$ /ml for Tomorrow's Sort
- Remainder of cells, $\sim 2 \times 10^8$ cells
- Spin/Decant, Freeze in 5 vials (4×10^7 /vial) at -80°C



Read & Understood by me,
James Forster

Date
 10/26/97

Invented by
James Forster
 Recorded by

Date
 9/8/97

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